THE CYTOCHROME SYSTEM IN MARINE LAMELLIBRANCH TISSUES [1]

KIYOZO KAWAI

Department of Zoology, University of Kyoto, Kyoto, Japan

The occurrence of the cytochromes and cytochrome oxidase, very similar to the oxidase of mammals, has been proved in certain marine molluscs (Ball and Meyerhof, 1940; Humphrey, 1947; Ghiretti-Magaldi, Giuditta and Ghiretti, 1957). However, it is still obscure whether the cytochrome system acts as a terminal oxidation system in their intact tissues. The mere presence of the cytochromes or cytochrome oxidase in a cell does not indicate to what extent the normal respiration is mediated actually through the cytochrome system. Recently it was suggested that the cytochrome system may not play a major role in the respiratory system of the oyster mantle, although the tissue contains cytochrome oxidase (Jodrey and Wilbur, 1955).

The present investigation was undertaken to throw some light on the connection of the cytochromes with the respiration of intact tissues of marine lamellibranchs. A portion of this work has been preliminarily reported (Kawai, 1958).

Materials and Methods

The respiratory studies were made at the Seto Marine Biological Laboratory during fall and early winter, and certain enzyme assays were carried out at the laboratory in Kyoto. Three species of marine lamellibranchs, the oyster, *Crassostrea gigas,* the pearl oyster, *Pinctada martensii* and the mussel, *Mytilus crassitesta,* were used as experimental materials. Specimens of the former two, each about two years old and about 11 and 6 cm. in shell height, respectively, were obtained from culture-farms in the vicinity of the Marine Laboratory, while *Mytilus*, about 8 cm. in shell height, was collected at a shore reef near the Laboratory.

Oxygen uptakes of intact tissues and extracts were measured at 25° C. in Warburg manometers, with vessels of about 9 ml. capacity. Respiratory measurements were carried out with several thin tissue pieces, 50 to 100 mg. in fresh weight, suspended in 1.5 ml. of sea water buffered at pH 8 with 0.03 *M* glycine (Robbie, 1946) or glycylglycine (Tyler and Horowitz, 1937). To absorb CO_2, 0.2 ml. of 0.5 *M* or 10% KOH and filter paper were placed in the center-well. In the cyanide experiments, NaCN was added to the main compartment and 0.2 ml. of KCN-KOH mixture (Robbie, 1946) was included in the center-well. Both glycine and glycylglycine of the concentration used had no effect on the respiration of the lamellibranch tissues. In the experiments of photo-reversibility of carbon monoxide inhibition, a 500-watt projector lamp was switched on at some

[1] Contributions from the Seto Marine Biological Laboratory, No. 334.

distance away from the water-bath. A slight absorption by the KOH solution in the center-well was subtracted from each manometric reading.

Absorption bands of cytochromes were observed with a low dispersion hand spectroscope and more accurate sites of the bands were measured with a Hilger wave-length spectrometer. The spectrophotometric studies of cytochrome oxidase were made with a Hitachi EPU-II spectrophotometer.

Carbon monoxide was prepared by decomposing formic acid with warm concentrated sulfuric acid. Cytochrome *c* was prepared from beef heart according to Keilin and Hartree (1945).

Results

1. *Cytochrome spectra*

The absorption spectra of the reduced cytochromes were examined on intact thin tissues or breis, packed about 2 mm. thick, adding a small amount of solid sodium hydrosulphite. The characteristic absorption bands corresponding to cytochromes $a + a_3$, *b* and *c*, could be clearly observed in the lamellibranch heart at room temperature. Cooling the heart with liquid-air by the method of Keilin and Hartree (1949), the bands were very intensified, being slightly shifted towards the violet. The sites of these cytochrome bands were estimated with the Hilger spectrometer at room temperature as follows: $a\alpha + a_3\alpha$: 603; $b\alpha$: 562; $c\alpha$: 550; $b\beta + c\beta$: 520–530 mμ.

In other tissues, such as gill, mantle, adductor muscle, etc., only the band of cytochrome *b* could be detected at room temperature. However, a feeble band of cytochrome $a + a_3$ and a lesser band of *c* appeared at liquid-air temperature. Table I

Table I

Observations on the α-bands of reduced cytochromes in marine lamellibranch tissues at liquid-air temperature

Animal	Tissue	Relative intensity of the absorption*		
		Cyt. $a+a_3$	Cyt. *b*	Cyt. *c*
Crassostrea gigas	Heart	+++	+++	++++
	Gill	++	+++	+
	Mantle	+	++	+−
	Adductor muscle	+	++	+
	Digestive diverticula	+	++	+−
Pinctada martensii	Heart	++	+++	+++
	Gill	+	++	+−
	Mantle	+	++	+−
	Adductor muscle	++	++	+
	Gonad	+	++	+−
	Digestive diverticula	−	++	+−
Mytilus crassitesta	Gill	+	++	+−
	Mantle	+−	++	−

* Very strong absorption is shown by the sign +++ or ++++, while very feeble band is indicated by +−. The sign − shows the absence of cytochrome band.

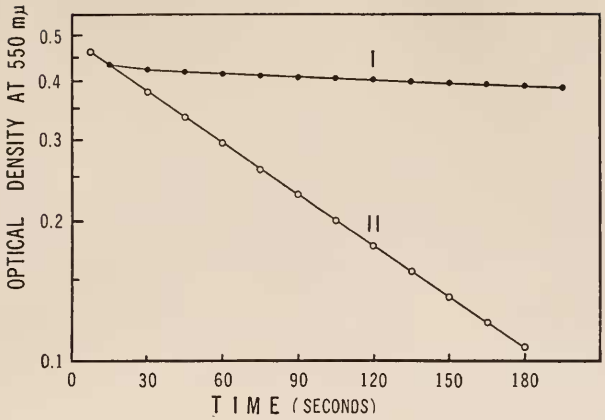

FIGURE 1. The oxidation of reduced cytochrome *c* by cytochrome oxidase from oyster gill. Reduced cytochrome *c* was prepared by reduction with a pinch of hydrosulphite and the excess hydrosulphite oxidized by shaking. Total volume 3 ml. Each cuvette contained final concentration of 2×10^{-5} *M* cytochrome *c*, 0.1 *M* phosphate buffer at pH 7.0 and 0.15 ml. of the extract. I contained 10^{-3} *M* cyanide; II, no cyanide. Reaction was followed at 25° C. The change of optical density was plotted on the logarithmic scale.

represents the results of these observations. The cytochromes are most abundant in the heart, where cytochrome *c* is predominant or equal to *b*. In other tissues, however, cytochrome *b* is apparently more dominant than $a + a_3$ or *c*. This fact forms a sharp contrast to the situation of the heart. Absence of the bands of $a + a_3$ or *c* in certain tissues may be due to their very low concentration. When a few drops of pyridine were added to the reduced tissues, a very intense pyridine hemochromogen band extending from about 550 to 560 mμ, with a mid-point at about 557 mμ, and a weak band lying about from 580 to 590 mμ were readily produced in all tissues examined. They are considered to be the absorption bands of pyridine derivatives of cytochromes *b* and *a* group, respectively.

2. *Cytochrome oxidase*

The enzyme activity was determined at 25° C. by two methods, *i.e.*, manometrically and spectrophotometrically. The extract for the enzyme study was prepared by homogenizing the excised tissue with a glass homogenizer in five parts of cold 1.24 *M* sucrose (isotonic with sea water) and squeezing through a thin cloth. The addition of an aliquot of the extract to a solution of reduced cytochrome *c* results in a rapid decrease in optical density at 550 mμ (Fig. 1). The activity of molluscan cytochrome oxidase is inhibited by about 94% in the presence of 10^{-3} *M* cyanide. The enzyme activity is also inhibited by carbon monoxide in the dark. Under the condition, 90% nitrogen + 10% oxygen in the control flask and 90% carbon monoxide + 10% oxygen in the experimental, the activity is inhibited by about 62% and the inhibition is completely eliminated by the illumination (Fig. 2).

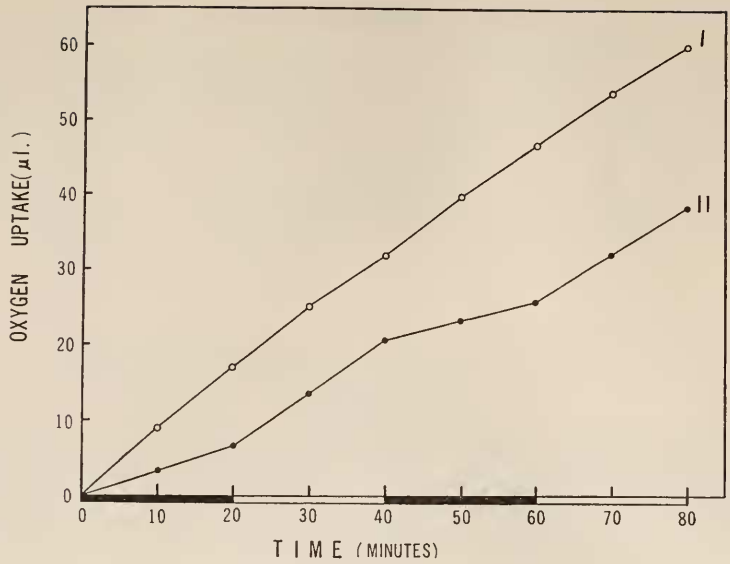

FIGURE 2. Effect of carbon monoxide on cytochrome oxidase from pearl oyster gill; 0.5 ml. extract in each Warburg flask. Final concentration: phosphate buffer at pH 7.0, 0.05 *M*; ascorbate, 0.01 *M*; cytochrome *c*, 2.2×10^{-5} *M*. Final volume 2.0 ml. I, in 90% N_2 + 10% O_2; II, in 90% CO + 10% O_2. Temperature 25° C. The black and white blocks under the base line show the periods of dark and light.

3. *Effect of carbon monoxide on the respiration of lamellibranch tissues*

Inhibition experiments were performed using the same gas mixture (CO/O_2 ratio of 9/1) as in the case of cytochrome oxidase. Controls with a gas mixture

TABLE II

Respiration of lamellibranch tissues in a gas mixture of 90% CO and 10% O_2

Animal, tissue	No. of determinations	O_2 uptake in control (μl./hr./100 mg. fresh wt.)	CO inhibition in darkness* (per cent)	Relative affinity constant**
Crassostrea gigas				
Heart	2	39	50	9.0
Gill	3	53	51	8.7
Mantle	2	29	53	8.0
Pinctada martensii				
Gill	4	48	47	10.1
Mantle	2	15	54	7.7
Digestive diverticula	1	27	46	10.6
Mytilus crassitesta				
Gill	2	26	52	8.3

* The complete elimination of CO inhibition by the illumination was observed in all tissues examined.

** Relative affinity constant of the tissue respiration for CO and O_2 was calculated by the Warburg equation. See text.

of nitrogen and oxygen (9/1) were also run, but there was no significant difference between oxygen uptakes in this control and in air.

Figure 3 shows the results of a typical experiment, obtained with the oyster mantle, indicating the presence of photo-reversibility of carbon monoxide inhibition. Nearly identical results, the inhibition of oxygen uptake in the CO gas mixture being about 50% in darkness and completely eliminated by light, were obtained in all tissues examined. These results are summarized in Table II. The relative affinity constants of the tissue respiration for carbon monoxide and oxygen were also calculated according to the equation of Warburg (1949); $K = n/1 - n \cdot pCO/pO_2$, where n is the fraction of oxygen consumption not inhibited, and pCO and pO_2 are, respectively, carbon monoxide and oxygen pressure. The values obtained, ranging from 7.7 to 10.6, are in good agreement with the average value of 8.2 reported for yeast cells in young cultures (Warburg, 1949). The stimulation effect of carbon monoxide on cell respiration, which has been found in certain marine eggs (Rothschild, 1949; Minganti, 1957; Rothschild and Tyler, 1958), was not observed in these lamellibranch tissues.

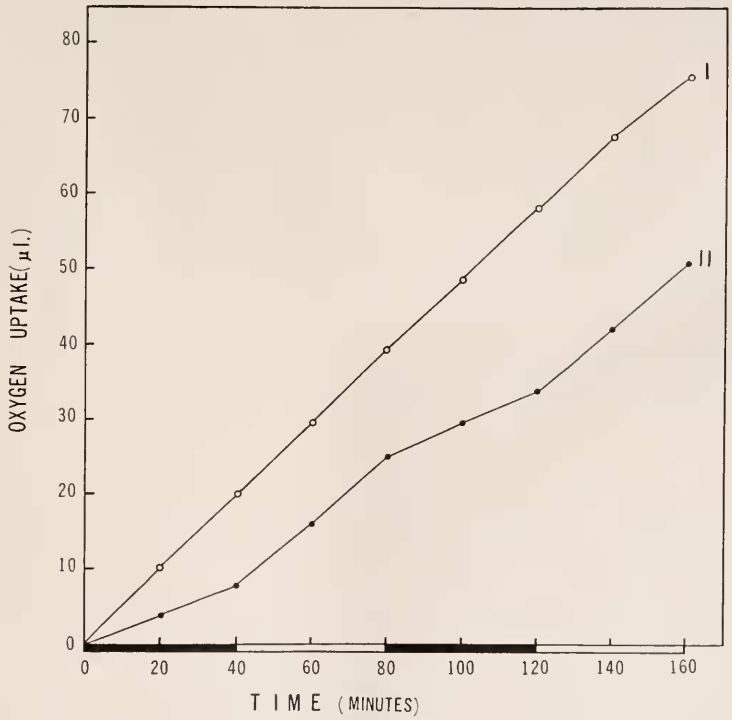

FIGURE 3. Effect of carbon monoxide on the respiration of oyster mantle. I, in 90% $N_2 + 10\%$ O_2; II, in 90% $CO + 10\%$ O_2. Each flask contained 100 mg. tissue pieces in 1.5 ml. of 0.03 *M* glycylglycine-buffered sea water. Temperature 25° C. The white blocks under the base line show the periods of illumination.

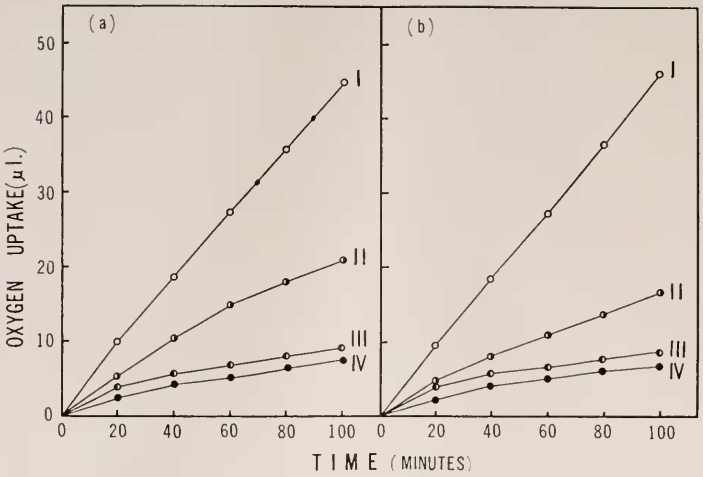

FIGURE 4. Effect of cyanide and methylene blue on the respiration of oyster tissues. (a) gill; (b) mantle. I, control; II, 10^{-3} *M* cyanide + 6×10^{-5} *M* methylene blue; III, 10^{-3} *M* cyanide + 10^{-5} *M* methylene blue; IV, 10^{-3} *M* cyanide. Each manometric flask contained 50 mg. of gill pieces or 100 mg. of mantle pieces in 1.5 ml. of sea water buffered with 0.03 *M* glycine. Manometric measurements were started twenty minutes after the tissues were immersed in each medium containing the reagents.

4. *Effect of cyanide on the respiration of oyster tissues*

The endogenous respiration of the oyster gill was depressed by 0.001 *M* cyanide to 15–20% of the control and the inhibition was partly restored to about 40% of the control in the presence of $6 \times 10^{-5}M$ methylene blue, or its nearly saturated solution in sea water; while a lower concentration of methylene blue, $1 \times 10^{-5}M$, was slightly effective to reverse the cyanide inhibition at an initial short period, but this effect completely disappeared within 40 to 60 minutes after the respiratory measurement was started (Fig. 4a). Very similar results, though the effect of $6 \times 10^{-5}M$ methylene blue was somewhat smaller, were also obtained in the cyanide inhibition of the oyster mantle respiration (Fig. 4b).

DISCUSSION

The marine lamellibranchs, *Crassostrea gigas*, *Pinctada martensii* and *Mytilus crassitesta*, used in this investigation have no oxygen carrier such as hemoglobin or hemocyanin in the blood. However, as reported here, their visceral cells possess a normal cytochrome system consisting of cytochromes *a*, *b*, *c* and a_3, *i.e.*, cytochrome oxidase. The absorption bands of cytochromes in their hearts being nearly equivalent to those in the cells of baker's yeast, it is inferred that the hearts probably contain as much cytochromes as yeast cells. Such high contents of cytochromes in the molluscan hearts, resembling mammalian hearts, may be concerned with their active movement. Although the contents of cytochromes are considerably low in other tissues, such as gill, mantle, adductor muscle, etc., there is apparently more cytochrome *b* than $a + a_3$ and *c*. This is very interesting when compared with the situation of the hearts, where cytochrome *c* is predominant or equal to *b*.

As the molluscan cytochrome oxidase shows photoreversibility in the carbon monoxide inhibition, it should contain iron atom, like the oxidase of mammals, in the active site. The relative affinity constant of the enzyme for carbon monoxide and oxygen, calculated from the equation of Warburg as described previously, is 5.5 under the condition employed. This value is very similar to that reported for cytochrome oxidase of sea urchin eggs (Krahl, Keltch, Neubeck and Clowes, 1941) and the spermatozoa of fresh-water mussels (Kawai and Higashi, 1959), but somewhat smaller than the value for mammalian cytochrome oxidase (Ball, Strittmatter and Cooper, 1951).

The presence of photoreversible inhibition of carbon monoxide in the respiration of lamellibranch tissues demonstrates that cytochrome oxidase acts as a terminal oxidase in these intact tissues. In a gas mixture of 90% carbon monoxide, the activity of molluscan cytochrome oxidase is inhibited by about 62% in the dark and the inhibition of the tissue respiration, though somewhat different in each tissue, is roughly 50%. Therefore, on the assumption that the oxidase *in vivo* is inhibited to the same extent in the extract, the respiration of intact tissues mediated through the cytochrome oxidase would be about 80% or more of the total respiration.

As is well known in many cells, autoxidizable redox dyes, exemplified by methylene blue, can restore the depressed respiration by cyanide. Such reversing effects, though not remarkable, could also be observed in the cyanide inhibition of oyster tissues using methylene blue (6×10^{-5} *M*) nearly saturated in sea water. According to Jodrey and Wilbur (1955), methylene blue, 1.4×10^{-5} *M* at the final concentration, was without appreciable effect in reversing the cyanide inhibition of the mantle respiration of the oyster (*Crassostrea virginica*). From this result they suggested that the cytochrome system may not play a major role in the oxidative metabolism of the oyster mantle. However, the ineffectiveness of methylene blue may be attributed to the matter of concentrations employed, because, in the present work, a lower concentration of methylene blue (1.0×10^{-5} *M*) was similarly ineffective to reverse the cyanide inhibition of the respiration of oyster tissues.

The author wishes to express his indebtness to Prof. D. Miyadi of Zoological Laboratory, Prof. S. Tanaka of Biochemical Laboratory and Dr. Y. Matsui, the Director of Nippon Institute for Scientific Research on Pearls, for their encouragements and numerous facilities given during this investigation. He is also indebted to Dr. R. Sato, Institute for Protein Research of Osaka University, for his kind advice concerning the spectroscopic examination, and to the Staff of the Seto Marine Biological Laboratory for their help and facilities offered. This investigation was supported, in part, by a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education.

Summary

1. The cytochrome system of the lamellibranchs, *Crassostrea gigas*, *Pinctada martensii* and *Mytilus crassitesta*, has been studied in relation to the respiration of intact tissues.

2. The visceral cells possess a normal cytochrome system consisting of cytochromes *a*, *b*, *c*, and a_3, *i.e.*, cytochrome oxidase. The oxidase is strongly inhibited

by cyanide and carbon monoxide. The carbon monoxide inhibition of the enzyme is completely eliminated by light.

3. Cytochromes are most plentiful in the heart, where cytochrome *c* is predominant or equal to *b*, while *b* is apparently more predominant than $a + a_3$ or *c* in other tissues.

4. In a gas mixture of 90% CO and 10% O_2, the respiration of various tissues is inhibited by about 50% in the dark and the inhibition is completely eliminated by the illumination. Cyanide, 0.001 *M*, depresses the respiration of the oyster tissues to about 15–20% of the control, and the inhibition is partly reversed in the presence of methylene blue (6×10^{-5} *M*) nearly saturated in sea water.

5. It is concluded that about 80% or more of the total respiration of intact lamellibranch tissues proceeds through the cytochrome system.

LITERATURE CITED

Ball, E. G., and B. J. Meyerhof, 1940. On the occurrence of iron-porphyrin compounds and succinic dehydrogenase in marine organisms possessing the copper blood pigment hemocyanin. *J. Biol. Chem.*, **134**: 483–494.

Ball, E. G., C. F. Strittmatter and O. Cooper, 1951. The reaction of cytochrome oxidase with carbon monoxide. *J. Biol. Chem.*, **193**: 635–647.

Ghiretti-Magaldi, A., A. Giuditta and F. Ghiretti, 1957. A study of the cytochromes of *Octopus vulgaris* Lam. *Biochem. J.*, **66**: 303–307.

Humphrey, G. F., 1947. The succinoxidase system in oyster muscle. *J. Exp. Biol.*, **24**: 352–360.

Jodrey, L. H., and K. M. Wilbur, 1955. Studies on shell formation. IV. The respiratory metabolism of the oyster mantle. *Biol. Bull.*, **108**: 346–358.

Kawai, K., 1958. Cytochrome system in oyster tissues. *Nature*, **181**: 1468.

Kawai, K., and S. Higashi, 1959. The cytochrome system in the spermatozoa of fresh-water mussels. *Seikagaku*, **31**: 97–101.

Keilin, D., and E. F. Hartree, 1945. Purification and properties of cytochrome c. *Biochem. J.*, **39**: 289–292.

Keilin, D., and E. F. Hartree, 1949. Effect of low temperature on the absorption spectra of haemoproteins: with observations on the absorption spectrum of oxygen. *Nature*, **164**: 254–259.

Krahl, M. E., A. K. Keltch, C. E. Neubeck and G. H. A. Clowes, 1941. Studies on cell metabolism and cell division. V. Cytochrome oxidase activity in eggs of *Arbacia punctulata*. *J. Gen. Physiol.*, **24**: 597–617.

Minganti, A., 1957. Experiments on the respiration of *Phallusia* eggs and embryos (ascidians). *Acta Embryo. et Morphol. Exp.*, **1**: 150–163.

Robbie, W. A., 1946. The quantitative control of cyanide in manometric experimentation. *J. Cell. Comp. Physiol.*, **27**: 181–209.

Rothschild, Lord, 1949. The metabolism of fertilized and unfertilized sea-urchin eggs. The action of light and carbon monoxide. *J. Exp. Biol.*, **26**: 100–111.

Rothschild, Lord, and A. Tyler, 1958. The oxidative metabolism of eggs of *Urechis caupo*. *Biol. Bull.*, **115**: 136–146.

Tyler, A., and N. H. Horowitz, 1937. Glycylglycine as a sea water buffer. *Science*, **86**: 85–86.

Warburg, O., 1949. Heavy Metal Prosthetic Groups and Enzyme Action. Clarendon Press, Oxord.